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Where is the Oxygen Binding Site of Cytochrome *c* Oxidase? Transmembrane Helices of Subunits I and II

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A set of hydrophobic free energy changes are reported for the 20 side chains in proteins derived from the accessible surface area of the side chain, its potential for hydrogen bond formation, and the energy expended in neutralizing the charge of acidic and basic groups. On this scale membrane-spanning segments appear to have HI $\gtrsim$ 1.20 kcal/residue in an 18-residue segment that is expected to be helical. The hydrophobicity profiles of human placental and yeast subunit I and beef heart and yeast subunit II of cytochrome *c* oxidase indicate 11 and two transmembrane helices probably are present in these subunits, respectively. Ligands of heme *a*, heme a_3, and Cu_b are probably in subunit I. However, histidine-376, which was proposed to be the proximal histidine ligand of heme a_3 by Welinder and Mikkelsen, is $\gtrsim$30 Å away from the region expected to bind heme a_3 and Cu_b. The heme a_3 site proposed by Wikstrom et al. in 1985 is more likely to be the site of heme *a*.

Introduction

Cytochrome *c* oxidase (CCO) with its four metal ion sites provides a challenging problem to inorganic chemists who are interested in understanding how metal ion sites function in biology. This is particularly true of the copper site Cu_a, which has properties unduplicated in small copper compounds such as a *g* value smaller than the free electron value.[1] This copper site may be evolutionarily related to the blue copper sites, which, like Cu_a, have the function of electron transfer and whose structures have been elucidated by crystallography,[2] but the physicochemical properties of Cu_a are distinct.[3] Compounds that can be considered models of the Cu_a site synthesized in the laboratory do not display the two spectroscopic properties associated with this site, its EPR parameters and the broad absorption band at ca. 830 nm.[4]

The challenge is no less true of the oxygen binding site in CCO, heme a_3–Cu_b, which is not observed by EPR spectroscopy as a result of the strong antiferromagnetic interaction between the high-spin Fe(III) ion of heme a_3 ($S = {}^5/_2$) and the paramagnetic Cu(II) ion ($S = {}^1/_2$) in the oxidized resting enzyme. This property also has not been duplicated in a copper–iron complex synthesized in the laboratory.[5] Extracted heme a^{3+}–bis(imidazole) was shown to be a very good model of the heme *a* site by EPR and MCD.[7a] Only the structure of this iron site, which transfers electrons from cytochrome *c* to the heme a_3–Cu_b site probably via Cu_a,[6] an octahedral low-spin Fe(III) ($S = {}^1/_2$) site with two axial imidazole ligands, is well understood.[7a]

The molecular formula of CCO from beef heart has been established by painstaking separation of the polypeptide chains that make up this enzyme and establishing the amino acid sequence of each chain.[8a,b] Twelve different chains (subunits) having a molecular weight of 202 787 make up CCO; this corresponds to the molecular weight of 1793 amino acids.[8a] CCO from rat liver may consist of 13 subunits.[9]

Electron microscopy of CCO crystals indicates that a dimeric structure exists in the membrane with each monomer forming a Y-shaped structure that protrudes from the lipid bilayer mostly on the cytoplasmic side.[10] There is indication that subunit III stabilizes dimer formation.[11] The split structure of the monomers leads to a large cleft in the dimer[10] and may form the high-affinity binding sites for cytochrome *c*.[12] The channel through which dioxygen travels from the matrix side to the heme a_3–Cu_b site is less clear. The oxygen binding site may be accessible to ligands from the matrix side of the membrane.[13] It is known that the porphyrin rings of the heme irons have an average orientation that is close to being perpendicular to the plane of the membrane.[14] This may suggest that the porphyrin rings are located at least partially in the lipid bilayer and may be attached to the transmembrane helices. Transmembrane helices anchor the large hydrophobic subunits I, II, and III in the membrane so that the

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largest of these, subunit I, does not seem to protrude very much from either the cytoplasmic or the matrix side of the lipid bilayer.[15] Subunit II has a large section of it exposed on the cytoplasmic side. Subunit III is also accessible from the cytoplasmic side.

Comparison of the nucleotide and amino acid sequences of bovine and human subunits I appears to indicate that hydrophobicity is a major chemical criterion in residue selection during evolution of CCO.[16] Several groups have predicted the number and regions of the transmembrane helices in various subunits of CCO using this criterion.[17] In this paper the transmembrane helices in subunits I and II of CCO from two very different sources are predicted by using a modified form of the von Heijne–Blomberg scale of hydrophobicity.[18] Eleven transmembrane helices are predicted in subunit I from yeast (*Saccharomyces cerevisae*) and human placental enzyme. The 18-residue helices predicted in subunit II of yeast and bovine CCO in this paper are in agreement with the reported lipid-buried segments of bovine CCO subunit II obtained from photocross-linking studies.[8c] Results obtained for subunit I are in agreement with Kyte and Doolittle[17a] with respect to the location of the nine transmembrane helices predicted by them in subunit I from yeast CCO using a scale based partially on the observed accessibilities of the side chains in proteins to solvent water. This raises questions about the location of the heme a_3–Cu_b site predicted to be located in subunit I by Mikkelsen and Welinder[19] and by Wikstrom and co-workers.[17d]

Procedure

Several hydrophobicity scales have been proposed from which the free energy gain upon removal of the side chains in proteins from contact with water can be estimated.[20] Of these the Nozaki–Tanford scale is based on the free energy of transfer of amino acids from water to an organic solvent such as ethanol.[20a] The linear relation observed by Chothia[21] between these free energies of transfer and the accessible surface areas of protein side chains led him to propose that about 25 cal of hydrophobic free energy is gained per Å^2 of surface area buried. In this work a hydrophobicity scale is derived in the same manner as von Heijne and Blomberg[18a] and, later, von Heijne,[18b] by using this relationship observed by Chothia.[21] The hydrophobic contribution to the free energy change when a protein side chain in random-coil conformation is removed from contact with water as the protein folds into a helical conformation was calculated from the accessible surface area of the side chain x in the tripeptide glycine-x-glycine.[21a] As in the von Heijne–Blomberg[18] and Engelman–Steitz[22] scales, corrections were made to the total free energy change of this process due to unfavorable contributions from hydrogen-bond loss and neutralization of charged groups. The average hydrogen-bond energy loss was estimated to be around +1.3 kcal as compensating hydrogen bonds are formed as the protein folds.[21] This gives more realistic hydrophobicity profiles of proteins than the use of the von Heijne–Blomberg scale, which results in unfavorable free energy changes upon protein folding except in highly hydrophobic transmembrane segments. Both von Heijne–Blomberg and Engelman–Steitz scales make different charge contributions for lysine, arginine, aspartic acid, and glutamic acid. Aspartic acid was taken as the model for a charged residue whose (unit) charge must be neutralized before the side chain can be transferred into the membrane. From the sum of the charge and polar contributions to the free energy change that occurs when aspartic acid is removed from contact with water from the free energy of transfer of acetic acid $\Delta G(H_2O \rightarrow \text{vapor})$ given by Wolfenden, Cullis, and Southgate,[20c] a contribution to the free energy change of +6.4 kcal per positive (or negative) charge was estimated. This correction was made for aspartic acid, glutamic acid, arginine and lysine. For histidine the estimated charge contribution of +1.4 kcal given by von Heijne and Blomberg[18] was used.

Table I. Hydrophobic (H), Hydrogen-Bond (H-bond), and Charge-Neutralization (charge) Contributions to the Total Free Energy Change (ΔG_H) Estimated in This Work When a Side-Chain in Random-Coil Conformation Goes into the Helical Conformation[18]

		contrib, kcal/side chain			ΔG_H, kcal/side
X^a	ASA,[b] Å^2	H-bond	charge	H	chain
A	40	0	0	−1.0	−1.0
R	150	+3.9	+6.4	−3.8	+6.5
N	85	+3.9	0	−2.1	+1.8
D	75	+2.6	+6.4	−1.9	+7.1
C	60	0	0	−1.5	−1.5
Q	105	+3.9	0	−2.6	+1.3
E	115	+2.6	+6.4	−2.9	+6.1
G	0	0	0	0	0.0
H	120	+2.6	+1.4	−3.0	+1.0
I	100	0	0	−2.5	−2.5
L	95	0	0	−2.4	−2.4
K	125	+2.6	+6.4	−3.1	+5.9
M	110	0	0	−2.8	−2.8
F	135	0	0	−3.4	−3.4
P	70	+2.6	0	−1.8	+0.8
S	40	+1.3	0	−1.0	+0.3
T	65	+1.3	0	−1.6	−0.3
W	180	+1.3	0	−4.5	−3.2
Y	155	+2.6	0	−3.9	−1.3
V	80	0	0	−2.0	−2.0

[a] Key A = alanine, R = arginine, N = asparagine, D = aspartic acid, C = cysteine or half-cystine, Q = glutamine, E = glutamic acid, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine, and V = valine. [b] ASA is the accessible surface area of a side chain in the tripeptide glycine-x-glycine where x is one of the 20 side chains.

Hydrophobicity profiles of subunits I and II of CCO were obtained as the sums of the ΔG_H values shown in Table I for overlapping segments of length 10 residues plotted against residue number. Very hydrophobic segments that may be membrane-spanning were apparent as high and wide peaks. As a segment of at least 18 residues corresponding to five turns of the α-helix is necessary to span the lipid bilayer,[23] the most probable transmembrane helix under each of the large hydrophobic peaks was taken to correspond to the 18-residue segment with the highest hydrophobicity sum. An 18-residue transmembrane segment has a hydrophobicity index HI >1.2 on this scale (HI = $-\Delta G_H$/residue and is a positive number for hydrophobic segments).[24] The most hydrophobic uncharged 18-residue segments in the soluble proteins listed by Levitt and Greer[25] have HI <1.0 with two exceptions: The most hydrophobic 18-residue segments in dogfish lactate dehydrogenase (for which the corrected amino acid sequence given by Eventoff et al.[26] was used) and liver alcohol dehydrogenase[25] have HI = 1.42 and 1.19, respectively. Kyte and Doolittle[17a] also report an average hydrophobicity in the range observed for membrane-spanning segments for dogfish lactate dehydrogenase. Some membrane-spanning segments in proteins may be difficult to predict. For example, in cytochrome b_5 there are two transmembrane helices by the C-terminus of the polypeptide chain.[27] Only the very hydrophobic

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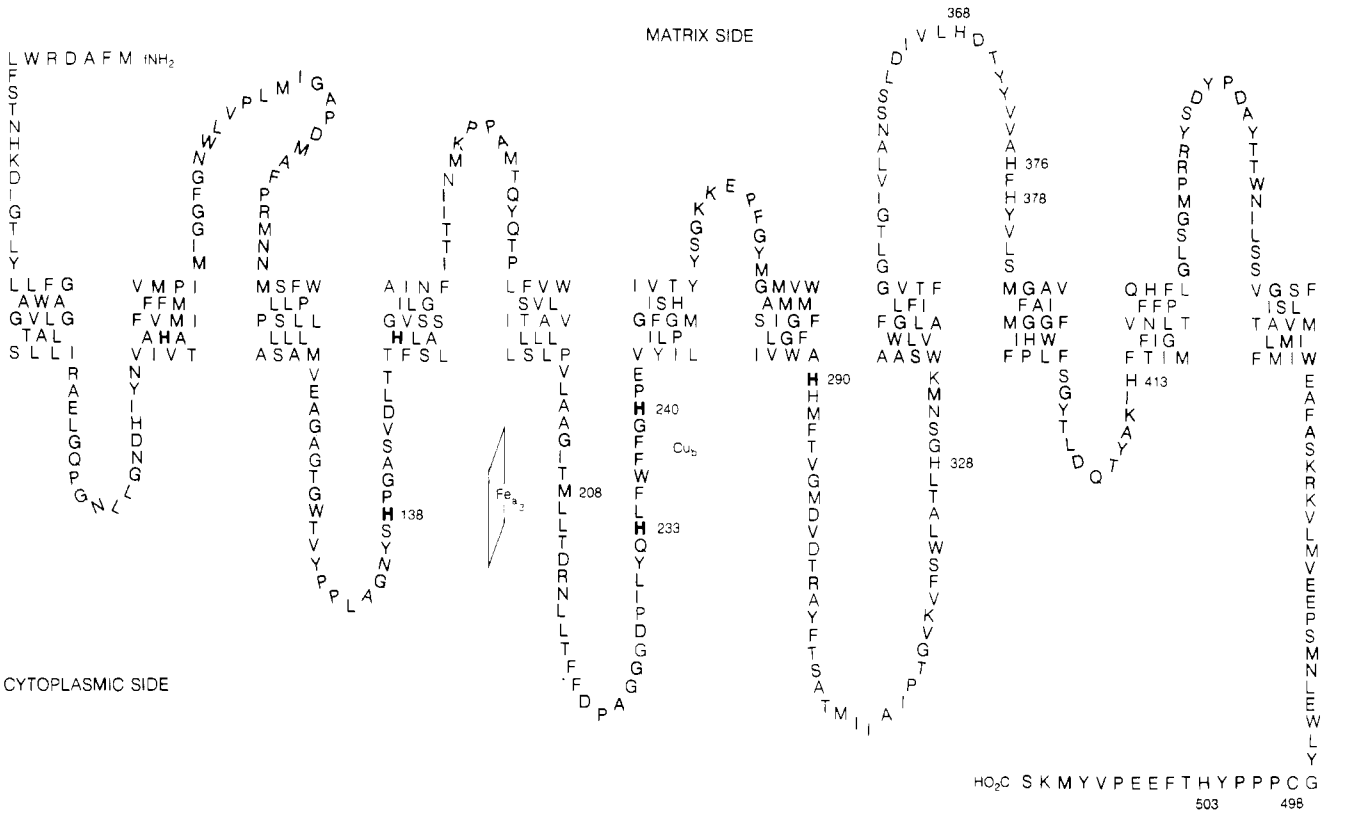

Figure 1. Transmembrane helices predicted to be present in subunit I of human placental subunit I. Very similar helices are predicted in yeast subunit I. The probable proximal and distal histidine ligands of heme-a (histidine-61 and histidine-151) are in boldface type in helices II and IV. The proximal histidine ligand of heme a_3 is histidine-138 in boldface type between helices III and IV. The probable histidine ligands of Cu_b are among the conserved histidines that have also been shown in boldface type and are numbered. The approximate positions of the porphyrin ring plane and Fe atom of heme a_3 and of the Cu_b ion of the proposed model of the oxygen-binding site of CCO are shown. The porphyrin ring of heme a_3 is probably partially immersed in the membrane.

final segment can be predicted to be membrane-spanning in this protein. The segment preceding this segment contains several charged acidic and basic residues (aspartate-86, arginine-88, lysine-90, lysine-92, glutamate-96). At least some of these must form salt bridges for this segment to be long enough to cross the membrane. Arginine-88 of the cyt b_5 side chain is long enough to remain in contact with solvent if it is close to the edge of the segment while aspartate-86 and aspartate-103 must be placed at the very edges of the segment for the carboxylate groups not to be buried.

Results

The ΔG_H values of the side chains in proteins shown in Table I are considered to be reasonable estimates of the free energy change upon removal of a side chain from contact with water in the folded protein. The sums of the ΔG_H values of overlapping 10-residue segments from the N-terminal to the C-terminal end of the protein chain indicate two transmembrane segments in subunit II and nine or more such segments in subunit I. For many of these segments HI is equal to or greater than the average hydrophobicity observed for membrane-spanning segments in proteins ($\approx$1.7 kcal/residue).[24] We suggest that segments listed fourth and sixth in subunits I from human and yeast CCO with HI $\geq$ 1.31 are membrane-spanning and the segment listed eighth, with HI = 1.18 (human) or 1.24 (yeast) is not because the former two segments have HI in the range observed for the membrane-spanning segments of some coat proteins, for example,[24] and are predicted to be transmembrane in five different species (bovine,[16] human,[16] yeast,[42a] *Neurospora crassa*,[42b] and *Drosophila melanogaster*[42c]) while the segment that is left out is not predicted in other species (HI $\leq$ 1.16). Thus the probable number of transmembrane helices in subunit I is 11. Notably, the first nine of these segments are predicted to be membrane-spanning by Kyte and Doolittle[17a] also, even though their hydropathy scale was obtained by very different assumptions.

Note that Wikstrom et al.,[17d] who used the Kyte–Doolittle scale with a "moving window" of 11 residues to obtain a hydropathy profile from which they obtained about 26-residue transmembrane helices, predict 12 transmembrane helices in the same regions as the 12 hydrophobic peaks we list in Table I. Their assignments place a negatively charged residue (glutamate-119) in the lipid

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Table II. Predicted Transmembrane (tm) Segments in Subunit I of CCO from Placental[16,40a] and Yeast[40b] Enzymes and Subunit II of CCO from Beef Heart[29a] and Yeast[46] Enzymes

segment	HI,[a] kcal/residue	tm helix	17a	19	17c	17d[b]	17b
			Subunit I (Placental)				
20–37	1.58	I		15–37	15–37	15–40	
56–73	1.92	II		56–75	56–80	55–80	
100–117	1.58	III		...	97–118	105–130	
147–164	1.35	IV		145–169	145–169	144–169	
or							
148–165	1.35						
183–200	2.19	V		183–210	181–206	183–213	
243–260	1.43	VI		...	...	241–264	
272–289	1.93	VII		268–289	267–289	272–297	
310–327	1.18	...		...	...	302–327	
334–351	1.85	VIII		338–359	334–359	338–363	
383–400	1.78	IX		371–390	...	377–402	
414–431	1.62	X		414–436	...	412–437	
456–473	1.97	XI		...	446–473	445–470	
			Subunit I (Yeast)				
19–36	1.68	I	14.36	15–37			
54–71	1.84	II	56–74	56–75			
100–117	1.61	III	99–117	...			
144–161	1.35	IV	146–164	145–169			
180–197	1.93	V	182–210	183–210			
or							
181–198	1.93						
or							
182–199	1.93						
242–259	1.31	VI	242–260	...			
269–286	1.73	VII	269–287	268–289			
308–325	1.24	...	...	...			
332–349	1.78	VIII	332–350	338–359			
375–392	1.61	IX	...	371–390			
412–429	1.93	X	...	414–436			
454–471	2.27	XI	451–469	...			
			Subunit II (Beef Heart)				
28–45	1.97	I	28–46		27–48		25–48
or							
29–46	1.97						
64–81	1.70	II	67—85		63–82		63–82
			Subunit II (Yeast)				
43–60	1.99	I	45–63				
87–104	1.91	II	87–105				

[a] HI is the average hydrophobicity of the 18-residue segment shown. [b] Bovine subunit I.

bilater in helix III while this work places this residue just outside the 19-residue third helix that corresponds to the lipid-buried portion of the transmembrane helix so that glutamate-119 is expected to be in the lipid head group region. Their assignments of 26-residue helices to span a 40-Å distance would allow lysine-319 to interact with the lipid phosphate groups. We place lysine-319 (conserved in all species) in a long connecting segment outside the transmembrane helices in Figure 1. With the assumption that the predicted transmembrane helices in subunit I shown in Figure 1 each cross the membrane once the N- and C-termini are placed on different sides of the membrane.

In subunit II two transmembrane helices place the short connecting segment on the matrix side of the membrane as subunit II contains the site of binding of cyt *c*, which is on the cytoplasmic side.[8c,17b] Subunit II is known to protrude from the membrane on the cytoplasmic side and can be labeled from this side.[15a] The 19-residue segments shown in Table I correspond to the lipid-buried sections of the longer transmembrane helices obtained by Bisson, Steffens, and Buse[8c] from their photocross-linking studies and are in general agreement with Capaldi et al,[17b] Kyte and Doolittle,[17a] and Bisson and Montecucco.[17c]

Discussion

The existence of two-subunit terminal oxidases is in agreement with one study in which both of the coppers and hemes of CCO were found located in subunits I and II.[29] Subunit II was proposed to be homologous to the blue copper proteins by Steffens and Buse[30a] and by Ryden and Lundgren.[30b] Capaldi et al[17b] proposed that cysteines-196 and -200, histidine-204, and histidine-161, all from subunit II of bovine CCO make up the Cu_a site as in the Chan model.[1b,31] Lundeen[32] proposed a Cu_a site of cysteine-196, cysteine-200, histidine-204, and methionine-207 (or threonine at this position in maize[33] or wheat[34]) with histidine-161 at a distance from this site. One of the two C-terminal cysteines of subunit II is missing in wheat, which has only cysteine-196 (bovine numbering), which makes the Chan model less likely while the presence of two or three sulfur ligands is consistent with a highly covalent Cu_a site.[35]

Some or all of the ligands of the metal centers other than Cu_a are probably in subunit I.[8c,17b,17d] Welinder and Mikkelsen[19] predicted the secondary structure of subunit I from five different species by three different methods and also obtained hydrophobicity plots for a "moving window" of seven residues using the Kyte–Doolittle scale. They considered three of the long hydrophobic segments they found not likely to be helical because these segments probably contained reverse turns and proposed that subunit I may have eight transmembrane helices. As shown in Table II their predicted transmembrane helices partially agree with those predicted in this work or in yeast subunit I by Kyte and Doolittle[17a] (also shown). Welinder and Mikkelsen's[19] proposed Cu_b site is in the vicinity of histidine-233 and histidine-240, both of which are conserved in all species, and is in the connecting segment between the predicted transmembrane helices V and VI in Figure 1 (helices IV and V in their work), which this work places

on the same side of the membrane as the C-terminus of subunit I (probably the cytoplasmic side). However, Welinder and Mikkelsen's[19] proposed heme a_3 site has, as its proximal histidine ligand, histidine-376, which, in their work, is placed in the second turn of helix in their predicted transmembrane helix VII while this work places histidine-376 in the connecting region between helices VIII and IX in Figure 1 on the side of the membrane as the N-terminus of subunit I. Thus, their proposed Cu_b and heme a_3 sites are separated by at least 30 Å, and this would not allow their binding oxygen as a peroxy derivative, a species trapped at low temperatures.[36] It would also not explain the observed S = 2 spin state of the Cu_b–heme a_3 site.[37] Kyte and Doolittle[17a] place the homologous histidines, histidine-232 and histidine-239, in yeast CCO also in the connecting segment between helices V and VI on the C-terminal side and place histidine-375 on the N-terminal side of subunit I. However, Bisson and Montecucco,[17c] who used the optimal matching hydrophobicity scale of Sweet and Eisenberg[20f] to predict the transmembrane helices in subunits I, II, and III of CCO from a number of species, predicted eight transmembrane helices in subunit I with histidine-233–histidine-240 and histidine-378 regions all on the same side of the membrane with these three histidines in the connecting segments between transmembrane helices (V–VI and VII–VIII in their work). Wikstrom et al.[17d] (who used the Kyte–Doolittle scale) place histidine-233 and histidine-240 between helices V and VI as we do; however, because they predict 12 helices and we predict 11, they place his-378 on the same side between their helices IX and X (our helices VIII and IX). However, Wikstrom et al.[17d] propose that the distal histidine ligand of heme a_3 is histidine-61 on the basis of their alignment of the subunit I sequence with leghemoglobin starting on the N-terminal end with histidine-151 as the proximal ligand. This is a probable assignment. Nevertheless, we consider the arguments of Welinder and Mikkelsen[19] based on the gene sequences in the globins and their observation that the histidine-233–histidine-240 region contains a cluster of conserved aromatic residues particularly relevant and think that this is the likely oxygen-binding site. Interestingly, the crystal structure of *Panulirus interruptus* hemocyanin reported recently by Gaykema et al.[38] shows each copper ion to be bound to three histidines, one of which is next to a highly hydrophobic pentapeptide containing two phenylalanines and one tryptophan. At the CCO proposed oxygen-binding site there is a pentapeptide with three phenylalanines and a tryptophan. The role these highly aromatic and hydrophobic pentapeptides may play in hemocyanin (an oxygen carrier) and cytochrome oxidase (which reduces O_2 to H_2O) may have particular relevance to oxygen-binding sites in biology. No endogenous ligand was detected in the X-ray study of the colorless hemocyanin crystals.[38] This dicopper site, like the heme a_3–Cu_b site, shows strong antiferromagnetic interaction of the two metal ions. We want to point out that in the very special environment of the oxygen-binding site of hemocyanin and the histidine-233–histidine-240 region in subunit I of CCO, which is probably the oxygen-binding site of CCO, the backbone carbonyl or the peptide bond may have a different electron distribution than is commonly observed. Under conditions in which an EPR signal is obtained for Cu_b of cytochrome oxidase, the EPR parameters indicate that Cu_b may have an oxygen ligand.[39] Backbone atoms in general avoid large aromatic residues and other backbone atoms of the same type and prefer to interact with backbone atoms not of the same type or small polar side chains that can form hydrogen bonds.[40] The crystal structure of hemocyanin was determined at 3.2-Å resolution, and therefore, an interaction of the type we envisage can not be demonstrated at this time but may be looked for if no endogenous ligand can be found at the methemocyanin site (there is no crystal structure of cytochrome oxidase).

To sum up, 11 transmembrane helices are predicted in subunit I of cytochrome oxidase based on the high hydrophobicity of these segments. The observation has been made that adjacent helices need not all be exceptionally hydrophobic to transverse the membrane.[41] The transmembrane helices predicted in this work are all very hydrophobic and are the most likely transmembrane segments. Figure 1 suggests that the oxygen-binding site of CCO is not buried in the lipid in agreement with Welinder and Mikkelsen,[19] who propose that several helices may form an oxygen pocket as in the globins. If his-233 is on the distal side of heme a_3, probable histidine ligands of Cu_b are among the conserved histidines in this region: his-233, his-240, his-290, and his-291. Histidine-291 is not conserved in *D. melanogaster* (frut fly)[42c] subunit I, but there is a histidine that may correspond to his-290 of bovine or human subunit I at position 288. It is not apparent in Figure 1 what side chains may provide sulfur donors to the Cu_b site. The single cysteine at 498 in human and bovine subunit I is not conserved in nonmammalian species. Methionines-208 and -292 are conserved in all species and are the only sulfur donors that may be in the vicinity according to Figure 1. These methionines and two of the histidines listed above may be the ligands of Cu_b in "pulsed" CCO, which has no bridging sulfur ligand to heme a_3 and does not exhibit an EPR signal due to Cu_b.[43] The probable axial ligands of the copper-depleted CCO heme a_3 site are conserved his-138 and met-208.[44] We propose that his-61 in transmembrane helix II and his-151 in transmembrane helix IV are the most likely axial ligands of the heme *a* iron and not of the heme a_3 iron as Wikstrom et al.[17d] proposed. The heme *a* porphyrin plane is tilted by about 30° from the normal to the plane of the membrane,[14] and this may arise from its being wedged between two transmembrane helices that are tilted on the average by about 10° or more from the normal to the plane of the membrane. In contrast, the heme a_3 plane is approximately normal to the plane of the membrane according to Erecinska, Blaisie, and co-workers.[14]

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